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Anthrax - Pasteur to the Present

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Few microbial pathogens have had as great an impact on the early development of medical bacteriology and immunology as Bacillus anthracis. From studies on this etiologic agent of anthrax during the mid-1800s have stemmed many of the fundamental concepts of infection and immunity now generally accepted. For example, Robert Koch's postulates concerning the etiology of disease were developed through his studies with anthrax. In 1881, Louis Pasteur and his assistants, Chamberland and Roux, performed their anthrax vaccine trials at Pouilly-le-Fort, thereby demonstrating the first successful use of attenuated bacterial vaccines. And, it was by implanting anthrax-infected tissues excised from rodents into the backs of frogs that Elie Metchnikoff made some of the earliest observations concerning phagocytosis (17).

Although anthrax has long been recognized as a disease primarily associated with herbivorous animals, it can occur in humans, usually as a result of incidental contact with infected animals or contaminated animal products. Descriptions of diseases consistent with anthrax were reported in early Hebrew, Greek, and Roman records, and the disease may have been the fifth Biblical plague of Egypt (Exodus, chpt. 9). From the 17th through the 19th centuries, anthrax in Europe caused enormous losses in domestic livestock and frequently manifested itself as Woolsorter's disease (pulmonary anthrax) among workers who handled contaminated wool in factories. It was in response to the major epidemics of the mid-1800s, that the French Minister of Agriculture commissioned Pasteur to develop methods to control

anthrax (6). In order to fully appreciate the rationale of Pasteur's approach to the problem and the genetic basis of his vaccine, it is necessary to present a general overview of the current understanding of the anthrax bacillus.

On culture media, such as blood or trypticase soy agar, B. anthracis grows as long chains of large, rod-shaped cells (1-1.25 by 3-5 um) in rough colonies with slightly serrated edges. However, growth on solid media (i.e., nutrient agar) containing 0.5% sodium bicarbonate in an atmosphere of 5-10% CO_2 induces capsule production; and smooth, convex, mucoid colonies with entire edges are formed. Capsule formation is also induced in vivo during the disease process, where it acts as a virulence factor by physically inhibiting phagocytosis of the vegetative cells. By virtue of its composition of poly-D-glutamate, the highly negative charge of the capsule also impedes phagocytosis through interference of complement opsonization (20). In addition to the capsule, the tripartite protein anthrax toxin is required for virulence, and like the capsule, its activities are directed toward inhibition of the host immune system. The term "anthrax toxin" is actually a misnomer in that there are two toxins. The edema toxin (ET) is composed of edema factor (EF, 89 kilodaltons, kDa) and a transport protein termed protective antigen (PA, 85 kDa)(2, 16). Lethal toxin (LT) is composed of lethal factor (LF, 83 kDa) and PA (1, 7). There is increasing evidence that when PA is bound to receptors on target host cells, the 85-kDa protein undergoes proteolytic activation by an as yet unidentified cell-surface protease (16). This activation yields a 20-kDa fragment, cleaved prior to the transport of EF or LF by the remaining 65 kDa fragment. Both in vivo and in vitro studies suggest that EF and LF either compete for the same PA binding site or mutually inhibit each other's transport through

allosteric hindrance (5,16). The edema toxin is an unusual bacterial extracytoplasmic adenylate cyclase which requires calmodulin for activity, a property usually associated only with mammalian adenylate cyclases (15). When intradermally injected into test animals (i.e., guinea pigs), ET produces edema; in vitro ET has been shown to inhibit neutrophil function (20). Lethal toxin, appears to inhibit and kill host macrophages (8). The precise biological or enzymatic basis for the toxicity of LT is not known. Fischer 344 rats that have been injected with LT expel froth, which originates from the lungs, through their nares and mouth immediately preceding death. The froth contains high concentrations of serum proteins but no blood cells, thereby supporting the premise that LT may alter pulmonic membrane integrity (7).

All three toxin proteins are coded for by a 110-megadalton plasmid, pXO1 (formerly designated pBA1) (18), whereas the capsule is encoded by a 60-megadalton plasmid, termed pXO2 (10, 26). Growth in the presence of novobiocin results in a preferential loss of the pXO2 plasmid, whereas growth of strains at elevated temperatures (i.e., 42-43°C) preferentially induces the loss of the pXO1 toxin plasmid. With the loss of either or both plasmids, there is a concomitant loss of virulence. It is from these findings that the genetic basis of Pasteur's attenuated anthrax vaccine has been deduced. In his initial studies, Pasteur attempted to attenuate the virulence of anthrax cultures by aging them, as he had done with chicken cholera cultures, but instead discovered that spores formed when cultures were grown at 37°C. Pasteur reasoned that because spores were metabolically dormant, as described by Koch, they would not become attenuated in the aging cultures. Recollection of his earlier findings that sporulation, but not growth, was inhibited at 42-43°C suggested a solution to this problem.

Pasteur and his assistants found that aging cultures at elevated temperatures with periodic passage to fresh media to maintain viability, yielded cultures attenuated in their virulence for test animals. Through a series of vaccine trials, Pasteur established that sheep were best protected when inoculated first with a highly attenuated culture, followed by a second inoculation 12 days later with less attenuated cultures. From the time of the public vaccine demonstration at Pouilly-le-Fort in the spring of 1881 to the early 1900s, these Pasteur-type anthrax vaccines were extensively used in cattle and sheep throughout Europe and the Americas, with dramatic decreases in animal mortality (6, 11). With numerous explanations offered over the past century to explain Pasteur's attenuation process, the current hypothesis states that by growing cultures at elevated temperatures to prevent sporulation, Pasteur induced the loss of the pXO1 toxin plasmid with a concomitant loss of virulence in the majority of bacilli present (6,19). As he increased the number of serial passages, the resultant cultures became more attenuated as the percentage of avirulent $pXO1^-$,$pXO2^+$ cells increased. Since pure cultures of $pXO1^-$,$pXO2^+$ cells are not protective (13), it is proposed that Pasteur's vaccine contained sublethal numbers of virulent, toxigenic bacilli that elicited antibodies to the PA toxin component, which provided protection. Evidence that Pasteur's vaccine did indeed contain virulent bacilli was provided by Pasteur himself. He demonstrated that cultures which were sufficiently attenuated for use as vaccines in cattle and sheep were nevertheless virulent in day-old guinea pigs. Pasteur then passaged attenuated cultures through day-old guinea pigs and found that their virulence was fully restored, thereby suggesting that such passage was selective for the virulent bacilli present. Perhaps Pasteur and his assis-

tants, Chamberland and Roux, should be given credit as the first bacterial geneticists to "cure" a bacterial strain of a plasmid.

Today, anthrax is rare in the U.S. and most developed countries due to routine vaccination of at-risk personnel and livestock and the enactment of strict public health laws. Although the true incidence of anthrax worldwide is not known (many cutaneous cases go unreported), recent estimates range between 20,000 and 100,000 cases annually. There have been several major outbreaks of human anthrax in the past decade. More than 6,000 cases occurred in Zimbabwe between October 1979 and March 1980 due in part to the breakdown of anthrax control measures during the country's internal conflicts. In April 1979, numerous deaths from pulmonary anthrax were reported shortly after an explosion at a research facility near Sverdlovsk, USSR. The incident is still shrouded in mystery; some reports have claimed that more than 1000 individuals died (21). In Thailand, 35 individuals developed oral-oropharyngeal and/or cutaneous anthrax after consumption of inadequately cooked meat from a water buffalo that had died of anthrax (22). And, in rural Paraguay (February 1987), 25 cases of cutaneous anthrax (2 fatalities) occurred among Lengua Indians following the slaughter and consumption of a diseased cow (12). Aside from these major outbreaks, small incidents or outbreaks are regularly reported, especially in areas where human contact with diseased animals is frequent, such as in southern Europe, the middle- and far-East, and Africa. Anthrax continues to threaten many endangered wildlife species, including the rhinocerous in southern Africa (25).

In humans, four forms of anthrax are now recognized: cutaneous, pulmonary, oral-oropharyngeal (22), and gastrointestinal. Only cutaneous anthrax will be discussed in detail here because it is the form of anthrax

most likely to be encountered by clinicians in developed countries. Cutaneous anthrax (approximately 90 - 95% of all anthrax cases) results from direct contact with infected animals or contaminated animal products or soil. In certain regions of the world, transmission by insect bites has been implicated (24, P. Turnbull, personal communication). Cutaneous lesions, usually on exposed areas of the body, may initially resemble insect bites. The lesion then progresses, showing rapid and extensive swelling due to the release of ET into the surrounding tissues. Lesions on the face, neck, and upper thorax may require intravenous administration of hydrocortisone (100-200 mg/day) to reduce respiratory obstruction resulting from the pressure of edematous tissues on the trachea (14, 23). In some cases, tracheostomy may be warranted (14). The center of the lesion eventually forms a black scab or eschar which can be several centimeters in diameter. Although B. anthracis is susceptible to virtually all routinely used antibiotics (14) except polymyxin B and neomycin (9), β-lactam antibiotics (i.e., penicillin) are the antibiotics of choice. After administration of penicillin, cutaneous lesions normally become sterile within 24 hr. In untreated cases, the mortality rate can be as high as 20%. Surgical intervention or tampering with the lesions is contraindicated and may result in spread of the infection, permanent scarring, and/or secondary infections. The edematous lesions are usually painless and, in treated cases, normally resolve in several weeks if kept clean, dry, and covered with a sterile loose-gauze dressing. Treatment of cutaneous anthrax has been recently reviewed (4, 14, 23).

The pulmonary form of the disease, which quickly becomes systemic, is almost always fatal due in part to the rapid progression of the disease and the insidious nature of the infection (there are no overt anthrax-specific

clinical symptoms until shortly before death). For these reasons, diagnosis of systemic anthrax infection is usually retrospective and is made at autopsy. Pulmonary infection results from inhalation of spores from contaminated materials (i.e., wool, hides). In experimental animals, spores do not germinate within the lungs, but rather, alveolar macrophages appear to clear the spores from the lungs and carry them through lymphatic channels to the sinusoids of regional lymph nodes. After spore germination, the resultant vegetative cells overgrow and escape the macrophage, invade the efferent lymphatics, and eventually enter the blood. The mononuclear phagocytic system, especially the spleen, serves as the principle defense against circulating bacilli, with pronounced splenomegaly a characteristic finding at necropsy. This line of defense is quickly overcome and bacteria are released into the blood, colonize secondary sites of infection and subsequently cause massive bacteremia, toxemia, and death.

Gastrointestinal and oral-oropharyngeal anthrax occur after consumption of inadequately-cooked, infected meat (22). The pathology of these two forms of anthrax is difficult to generalize in that ingested, contaminated foodstuffs may contain various combinations of spores, vegetative cells, and toxins, and consequently, variations occur in their clinical presentation. As with cutaneous lesions around the face, neck, and upper thorax, treatment of oral-oropharyngeal lesions may require tracheostomy and administration of hydrocortisone in addition to antibiotic therapy. In approximately 25 to 35% of untreated cases, gastrointestinal infections become systemic with a correspondingly high mortality rate. These figures are, however, rather speculative, since the true incidence of this form of anthrax is unknown.

Bacillus anthracis is a facultative, spore-forming, Gram-positive bacillus, which is closely related to B. cereus, B. thuringiensis, and B. mycoides with respect to DNA homology, surface antigens, and biochemical reactions. For these reasons, the most reliable methodologies for identifying B. anthracis are based on demonstration of its toxin components; detection of its unique galactose/N-acetylglucosamine polysaccharide, with monoclonal antibody or lectin based assays; and use of specific, lysogenic bacteriophage (3, 25). Although less specific, microscopic examination, capsule staining, and culture characteristics are also used in presumptive identification. In endemic areas of the world, the clinical presentation of the cutaneous form of anthrax is almost exclusively used for diagnosis. In collaborative, retrospective investigations of recent anthrax outbreaks, I have shown that patients with cutaneous, oral-oropharyngeal, or gastrointestinal anthrax have significant antibody titers to PA and LF as determined by enzyme-linked-immunospecific assays (ELISA). Unfortunately, in cases where antibody titers are low, there exists a "gray zone" between positive and negative sera, which often makes the ELISA assay difficult to interpret. However, through the use of purified PA and LF and electrophoretic-immuno-transblots (EITB, Western blots), this "gray zone" has been virtually eliminated. For example, in the Paraguay outbreak, sera from 11 of 12 cases were positive by EITB (92% sensitivity) whereas none of the 18 controls were positive (100% specificity) (12). For further descriptions of B. anthracis and recent developments in methodologies to differentiate it from closely related species, the I refer you to the literature (3, 25).

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